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I, the below-named translator, hereby declare:

(1) That my name, mailing address and citizenship are as stated below;

(2) That I am knowledgeable in the English language and in the Korean language in which Korean Patent Application No. 1999-27418 was filed on July 8, 1999; and

(3) That I have translated said Korean Patent Application No. 1999-27418 into English, which English text is attached hereto, and believe that said translation is a true and complete translation of the aforementioned Korean patent application.

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FILING DETAILS OF THE PATENT APPLICATION

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[TITLE OF THE INVENTION]
MODIFIED HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR AND PROCESS FOR PRODUCING SAME

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The above application is filed in accordance with Article 42 of Korean Patent Law, and the request for the examination of the above application is filed in accordance with Article 60 of Korean Patent Law.

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Abstract

The present invention relates to a modified human Granulocyte-Colony Stimulating Factor (hG-CSF); a gene encoding the said peptide; a vector comprising the said gene; and a microorganism transformed with the said vector. Also, the present invention relates to a process for producing a modified hG-CSF having no added methionine residue at its N-terminus which comprises transforming *E. Coli.* with the vector comprising the gene encoding the modified hG-CSF and a gene encoding signal peptide attached at 5'-end thereof; and culturing the transformed *E. Coli.* under an appropriate condition to periplasm.

Representative Drawing

Fig. 1

Specification

Title of the Invention

MODIFIED HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR AND PROCESS FOR PRODUCING SAME

Brief Description of Drawings

Fig. 1 represents the nucleotide and amino acid sequences of the wild-type human granulocyte-stimulating factor composed of 174 amino acid residues (SEQ ID NOS: 1 and 2).

Fig. 2 represents the procedure for constructing the plasmid pT-CSF.

Fig. 3 represents the procedure for constructing the plasmid pT14S1SG.

Fig. 4 represents the procedure for constructing the plasmid pT14SS1SG.

Fig. 5 represents the procedure for constructing the plasmid pT140SSG-4T22Q.

Fig. 6 represents the procedure for constructing the plasmid pT14SS1S17SEG.

Fig. 7 represents the procedure for constructing the plasmid pT01SG.

Fig. 8 represents the procedure for constructing the plasmid pBADG.

Fig. 9 represents the procedure for constructing the plasmid pBAD2M3VG.

Fig. 10a and 10b represent the results of Western blot analysis, which confirm the expression of hG-CSF and modified hG-CSFs from recombinant cell lines and the molecular weight of expressed proteins, respectively.

Fig. 11 represents the cellular activities of hG-CSF and modified hG-CSF produced from recombinant cell lines.

Detailed Description of the Invention

Objective of the Invention

Field of the Invention and Prior Art

The present invention relates to a modified human granulocyte-colony stimulating Factor (hG-CSF) and a process for producing same. Specifically, the present invention relates to a modified hG-CSF; a gene encoding the said peptide; a vector comprising the said gene; a microorganism transformed with the said vector; and a process for producing the modified hG-CSF having no added methionine residue at its N-terminus to periplasm which comprises transforming *E. Coli.* with the vector comprising the gene encoding the modified hG-CSF and a gene encoding signal peptide attached at 5'-end thereof; and culturing the transformed *E. Coli.* under an appropriate condition.

The term colony stimulating factor (CSF) has been known to be produced by the cells such as T-cell, monocytic macrophage, fibroblast, and epithelial cell; and these cells are normally distributed generally throughout the body. CSF is inclusive of granulocyte/macrophage-colony stimulating factor (GM-CSF) which stimulates stem cells of granulocyte or macrophage to induce the differentiation thereof and proliferation of granulocyte or macrophage colonies and macrophage-colony stimulating factor (M-CSF) which induces the formation of the colonies of monocytic macrophage colony. Further, there is a granulocyte-colony stimulating factor(G-CSF) which induce the formation of the colonies of granulocyte.

In vivo, G-CSF induces the differentiation of bone marrow leucocytes and enhances the function of mature granulocyte and, accordingly, it's clinical importance in treating leukemia has been well established.

Human G-CSF(hG-CSF) is a protein consisting of 174 or 177 amino acids, the 174 amino-acid variety having higher neutrophil-enhancing

activity(Morishita, K. et al., J. Biol. Chem., 262, 15208-15213(1987)). The amino acid sequence of hG-CSF consisting of 174 amino acids is shown in Fig. 1 and there have been many studies for the mass production of hG-CSF by manipulating a gene encoding said hG-CSF.

So far, in order to isolate and purify G-CSF, the cytoculturing method comprising the steps of culturing the cells and isolating G-CSF from the supernatant. However, this gives a product of low concentration; thus, it required a complicated purification process in order to obtain a trace amount of G-CSF from a large quantity of the culture solution.

For instance, Chugai Pharmaceuticals Co., Ltd. (Japan) has disclosed the amino acid sequence of hG-CSF and a gene encoding same (Korean Patent Publication Nos. 91-5624 and 92-2312), and reported a method for preparing proteins having hG-CSF activity by a gene recombination process (Korean Patent Nos. 47178, 53723 and 57582). In this preparation method, glycosylated hG-CSF is produced in a mammalian cell by employing a genomic DNA or cDNA comprising a polynucleotide encoding hG-CSF. The glycosylated hG-CSF has an O-glycosidic sugar chain, but, it is known that said sugar chain is not necessary for the activity of hG-CSF(Lawrence, M. et al., Science, 232, 61(1986)). Further, it is also well-known that the production of glycosylated hG-CSF employing mammalian cells requires expensive materials and facilities, and therefore, such a process is not economically feasible.

Meanwhile, there have been attempts to produce non-glycosylated hG-CSF by employing a microorganism, e.g., *E. coli.* In these studies, hG-CSFs having 175 or 178 amino acids having a methionine residue attached at the N-terminus thereof are obtained due to the ATG initiation codon employed in the microorganism. The additional methionine residue, however, causes undesirable immune responses in human body when the recombinant hG-CSF is administered

thereto(European Patent Publication No. 256,843). Further, most of the methionine-containing hG-CSFs produced in *E. coli* are deposited in the cells as insoluble inclusion bodies, and they must be converted to an active form through a refolding process, at a significant loss of yield. In this regard, four of the five Cys residues present in wild-type hG-CSF participate in forming disulfide bonds, while the remaining one contributes to the depression of a folding titer and stability of the protein in solution. Further, the glycosylated hG-CSF obtained by culturing a mammalian cell has O-glycosidic sugar chain and it inhibits the aggregation at high concentration even though sugar is not necessary for hG-CSF activities. However, the non-glycosylated hG-CSF expressed in *E. Coli.* easily aggregates at high concentration due to low solubility.

Recently, in order to solve the problems associated with the production of a foreign protein within a microbial cell, various efforts have been made to develop a method based on efficient secretion of a target protein across the microbial cell membrane into the extra-cellular domain.

Internal host production method is a way of producing the targeted protein in the host's cytoplasm through which high production level can be achieved. However, melatonine is added to the N-terminus of the natural amino acid sequence of the accumulated protein in the cytoplasm, and the recombinant protein adopts an insoluble form and after extraction, it easily adopts a structure that is higher than its natural form. As a result, the internal-host production requires a refolding process to change the protein's high degree structure to its wild type form, plus, this means that it requires an additional complicated step in the manufacturing process.

On the other hand, in the secretory production method, the desired recombinant protein manifests itself as a fusion protein wherein a signal peptide is added to the N-terminus of the protein. When this fusion protein permeates

through the cell membrane, the signal peptide is removed due to an enzyme in E. Coli. and the desired protein in its wild type structure is secreted. The secretory production method is advantageous in that we can obtain a resultant protein whose amino acid sequence is identical to the wild type. However, the yield of a secretory production method is often quite low due to unsatisfactory efficiencies in both the membrane transport and the subsequent purification process. Especially, if mammalian protein is secreted through prokaryotes then it is well known that the product yield is very low compared to secretion of prokaryotic protein; therefore, a superior secretory production method is desired.

Recently, it has been made known of secretion of protein in microorganisms, and research is in progress to find an efficient secretory production of protein. A signal peptide research is one of those studies being done. In secreting protein inside microorganisms, as mentioned above, it requires the fusion protein to permeate the cell membrane and be precisely removed.

In the signal peptides, it is known that the positive charge area of the N-terminus electrostatic charge field and a number of the central hydrophobic field are important, and researches are being conducted on the subjects. Wotaka et al., have reported that in a secretory production method using as a host, a modified signal peptide can be efficiently prepared by adding basic amino acid Argenine (Arg) to the N-terminal region of the signal peptide of the middle outermabrane protein in *Bacillus brevis*; and adding hydrophobic amino acid Leucine(Leu) to the central region thereof (Japanese Patent Publication No. Pyung 7-51072). However, there are no guiding principles for this plasmodium of a signal peptide, and a desirable signal peptide must be found through a trial and error.

Consequently, a method for mass production through a microorganism of soluble hG-CSF who lacks the addition of methionine residue on the amino

terminus is required. For instance, in a method employing a signal peptide, a desired protein is expressed in the form of a fusion protein wherein a signal peptide is added to the N-terminus of the protein. When the fusion protein passes through the cell membrane, the signal peptide is removed by an enzyme and the desired protein is secreted in a mature form. The secretory production method is advantageous in that the produced amino acid sequence is usually identical to the wild-type. However, the yield of a secretory production method is often quite low due to unsatisfactory efficiencies in both the membrane transport and the subsequent purification process. This is in line with the well-known fact that the yield of a mammalian protein produced in a secretory mode in prokaryotes is very low: Hitherto, no microbial method has been reported for the efficient expression and secretion of soluble hG-CSF having no added methionine residue at its N-terminus.

The present inventors have previously reported the use of a new secretory signal peptide prepared by modifying the signal peptide of *E. coli* thermoresistant enterotoxin II(Korean Patent Application No. 98-38061) in the production of hG-CSF. Specifically, an expression vector comprising a hG-CSF gene attached to the end of the modified signal peptide of *E. coli* thermoresistant enterotoxin II was prepared, and biologically active, mature hG-CSF was expressed by employing *E. coli* transformed with the expression vector. However, most of the expressed hG-CSF accumulated in the cytoplasm rather than in the periplasm. Therefore, the present inventors have confirmed that a use of signal peptide of the *E. Coli.* thermoresistant enterotoxin II increases the expression level of hG-CSF but the aspect of expression is similar to the production in cytoplasm.

Therefore, the present inventors have endeavored further to develop an efficient secretory method for the production of hG-CSF in a microorganism and have found that a modified hG-CSF, which is prepared by replacing at least one

amino acid residue, especially, the 17th cysteine residue, of wild-type hG-CSF with other amino acid, retains the biological activity of the wild-type, and that the modified hG-CSF having no methionine residue at the N-terminus thereof can be efficiently expressed and secreted by a microorganism when an appropriate secretory signal peptide is employed.

Objective of the Invention

Accordingly, it is an object of the present invention to provide a modified human granulocyte-stimulating factor(hG-CSF) which can be efficiently produced using a microorganism..

It is another object of the present invention to provide a DNA encoding said peptide and a vector comprising said gene.

It is a further object of the present invention to provide a microorganism transformed with said vector.

It is a still further object of the present invention to provide a process for producing a hG-CGF which is non-attached methionine residue to amino terminus using said microorganism.

Constitution and Function of the Invention

In accordance with one aspect of the present invention, there is provided a modified hG-CSF which can be efficiently produced, a gene encoding said peptide, a vector comprising said gene and a microorganism transformed with said vector.

The modified hG-CSF of the present invention is the hG-CGF non-attached methionine residue to amino terminus which having a character in that at least one of the 1st, 2nd, 3rd and 17th amino acids of wild-type hG-CSF consisted of 174 amino acids(fig. 1) is replaced by another amino acid.

Specifically, it is desired that these amino acids: 1st amino acid as threonine; 2nd amino acid as proline and 3rd amino acid as lycine; 17th amino acid as cysteine; 1st amino acid as threonine and 17th as cysteine; and 2nd amino acid as proline; 3rd amino acid as lycine and 17th as cysteine, each be replaced by a different amino acids. More preferred are those obtained by replacing the 17th amino acid of hG-CSF with an amino acid which is uncharged at neutral pH. The modified hG-CSF of the present invention is in the most desirable form:

a) the 1st amino acid threonine is replaced by serine ([Ser^1] hG-CSF);
b) the 2nd amino acid proline is replaced by methionine, and 3rd amino acid lycine is replaced by valine ([Met^2, Val^3] hG-CSF);
c) the 17th amino acid serine is replaced by threonine, alanine, or glycine ([Ser^{17}, Thr^{17}, Ala^{17}, or Gly^{17}] hG-CSF);
d) the 1st amino acid threonine and 17th amino acid cysteine are both replaced by serine ([Ser^1, Ser^{17}] hG-CSF); and
e) the 2nd amino acid proline is replaced by methionine, 3rd amino acid lycine is replaced by valine, and 17th amino acid cysteine is replaced by serine ([Met^2, Val^3, Ser^{17}] hG-CSF). The 32 N-terminal amino acids: [Ser^1, Ser^{17}] hG-CSF; [Ser^{17}] hG-CSF; [Ser^{17}] hG-CSF; [Thr^{17}] hG-CSF; [Ala^{17}] hG-CSF; [Gly^{17}] hG-CSF; [Met^2, Val^3] hG-CSF; and [Met^2, Val^3, Ser^{17}] hG-CSF sequence has sequence numbers 56, 58, 60, 62, 64, 66, 68, and 70, and the amino acid sequence of the rest is identical to the wild type hG-CSF.

Four of the five Cys residues of hG-CSF participate in forming disulfide bonds, while the 17th Cys residue remains unbonded in the natural state. However, when a large amount of hG-CSF is expressed in recombinant cells, the

17th Cys residue gets involved in inter-molecular disulfide bond formation, leading to the accumulation of agglomerated hG-CSFs in the cytoplasm. However, the inventive modified hG-CSF having an amino acid other than Cys at the 17th position is free of such problem and can be effectively produced by a secretory method using an appropriately transformed microorganism. Also, it is judged that the residues on the amino-terminus will adopt a free form and will not directly affect a protein's third dimension; thus, in order to increase the efficiency of the secretion, other residues can replace the residues on the amino-terminus.

In the present invention, the above modified hG-CSF is manufactured, and the modified hG-CSFs indicative of the present invention, that is, the genes that codes the N-terminus 32 amino acid [Ser^{1}] hG-CSF; [Met^{2}, Val^{3}] hG-CSF; [Ser^{17}] hG-CSF; [Thr^{17}]hG-CSF; [Ala^{17}]hG-CSF; [Gly^{17}]hG-CSF; [Ser^{1}, Ser^{17}]hG-CSF; and [Met^{2}, Val^{3}, Ser^{17}]hG-CSF with sequence numbers 55, 57, 59, 61, 63, 65, 67, and 69 possess a basic sequence, after which the rest of the sequence is identical to the wild type hG-CSF.

The modified hG-CSF of the present invention may be encoded by a gene comprising a nucleotide sequence deduced from the modified hG-CSF amino acid sequence according to the genetic code. It is known that several different codons encoding a specific amino acid may exist due to the codon degeneracy, and, therefore, the present invention includes in its scope all nucleotide sequences deduced from the modified hG-CSF amino acid sequence. Preferably, the modified hG-CSF gene sequence includes one or more preferred codons of *E. coli*.

The gene thus prepared may be inserted to a conventional vector to obtain an expression vector, which may, in turn, be introduced into a suitable host, e.g., an *E. coli*. The expression vector may further comprise a signal peptide. Representative signal peptides include a thermoresistant E. coli. enterotoxin II signal peptide(SEQ ID NO: 53), a modified thermoresistant E. coli enterotoxin II

signal peptide(SEQ ID NO: 54), a beta lactamase signal peptide(SEQ ID NO: 24), Gene III signal peptide(SEQ ID NO: 42) or modified peptide thereof, but these do not limit the signal peptides which may be used in the present invention. The promoter used in preparing the expression vector of present invention may be any of those which can express a heterologous protein in a microorganism host. Specially, lac, Tac, and arabinose promoter is preferred when the heterologous protein is expressed from *E. coli.*

The expression vectors of the present invention may be introduced into microorganism, eg., *E. Coli.* BL21(DE3) (Novagen), *E. Coli.* XL-1 blue (Novagen) to manufacture transformants. Examples of such transformants are: *E. Coli.* BL21(DE3)/pT14SS1SG (HM 10310), *E. Coli.* BL21(DE3)/pT14SS1S17SEG (HM 10311; No. KCCM-10154), *E. Coli.* BL21(DE3)/pT01SG (HM10409), *E. Coli.* BL21(DE3)/pT01S17SG (HM 10410; No. KCCM-10151), *E. Coli.* BL21(DE3)/pT017SG (HM 10411; No. KCCM-10152), *E. Coli.* BL21(DE3)/pT017TG (HM 10413), *E. Coli.* BL21(DE3)/pT017AG (HM 10414), *E. Coli.* BL21(DE3)/pT017GG (HM 10415), *E. Coli* BL21(DE3)/pBAD2M3VG (HM 10510; No. KCCM-10153), *E. Coli.* BL21(DE3)/pBAD17SG (HM 10511) or *E. Coli.* BL21(DE3)/pBAD2M3V17SG (HM 10512).

Also, in the present invention, *E. Coli.* is transformed by an expression vector, which comprises a modified hG-CSF encoding gene and a signal peptide gene connected to its 5'-end. Thus, the present invention provides the secretory production method where a transformed *E. Coli.* is cultivated under an appropriate condition and by which a modified hG-CSF lacking a methionine is secreted through the periplasm of the *E. Coli.* The modified hG-CSF gene; a signal peptide gene; a hG-CSF expressing vector; and a transformed *E. Coli.* by the said vector, which can be used in the present invented method are as described

above.

According to the secretory production method of the present invention, when using the transformed *E. Coli.*, it is possible to produce by secretion hG-CSF at a level higher than 1l/g of the culture medium, and the resultant hG-CSF has biological activity equal or higher than that of the wild type hG-CSF in the cytoplasm.

The following examples are intended to further illustrate the present invention without limiting its scope.

Example 1: Preparation of a Gene Encoding hG-CSF

A cDNA gene encoding hG-CSF was prepared by carrying out PCR using as an hG-CSF template. The primers used are those described in US Patent No. 4,810,643. For example, one can use the above US patented synthetic oligonucleotide primer, and hG-CSF gene can be amplified using hG-CSF basic sequence as a template through PCR.

A signal peptide is included at the front of the resultant hG-CSF cDNA; thus, in order to delete this, a vector pUC19-G-CSF (Biolabs, USA) was subjected to PCR using the primers of SEQ ID Nos. 3 and 4. The primer of SEQ ID No. 3 was designed to provide an Nde I restriction site (5'-CATATG-3') upstream from the first amino acid (threonine) codon of a mature hG-CSF, and the primer of SEQ ID No. 4, to provide a BamHI restriction site (5'-GGATCC-3') downstream from the termination codon thereof. Subsequently, the amplified fragmented gene was cleaved with a restriction enzyme NdeI and BamHI to obtain a gene encoding a mature hG-CSF. Also, one can manufacture a pT-CSF plasmid that contains hG-CSF signal peptide deleted hG-CSF gene by restricting a pET-14B plasmid (Novagen) using restriction enzymes Nde I and BamHI, then inserting the above mentioned DNA fragment between Nde I/BamHI restriction

sites. Figure 2 illustrates the said manufacturing process.

Example 2

a) Construction of a vector containing the gene encoding *E. Coli* enterotoxin II signal peptide and a modified hG-CSF.

Using the combined oligonucleotide having SEQ ID Nos. 5 and 6, a DNA fragment that contains an enterotoxin signal peptide is manufactured. The above oligonucleotide is manufactured by including restriction enzyme NcoI and its complementary restriction enzyme BspHI on the ATG initiation codon of the enterotoxin signal peptide; and by altering only the codon, and not the amino acid on the 3'-end so that a Mlu I restriction enzyme recognition site is introduced to it. By inserting this DNA fragment into the SmaI segment of the plasmid pUC19, a restriction enzyme BspH I recognition site exists on the amino end of the enterotoxin signal peptide. It also created a plasmid pUC19ST that possess a restriction enzyme Mlu I recognition site on the carboxyl end of the said signal peptide.

Also, in order to connect the enterotoxin signal peptide with the modified hG-CSF gene, using the plasmid pT-CSF from the example 1 as a template, a hG-CSF DNA fragment, which contains Mlu I and BamHI terminal, is manufactured. Specifically, the first amino acid, threonine, of hG-CSF is replaced by serine, and using the oligonucleotide primers of SEQ ID Nos. 7 and 8, which are designed to insert the restriction enzyme BamHI recognition sequence (5'-GGATCC-3') after the termination codon, a DNA fragment is obtained that contains the modified hG-CSF [Ser1] hG-CSF encoding sequence through PCR; and the said fragment is cleaved by restriction enzymes Mlu I and BamHI. On the other hand, the plasmid pUC19ST is restricted by the restriction enzyme Mlu I, and then digested

using the restriction enzyme BamHI; thus, a vector containing Mlu I and BamHI ends is obtained. Connecting the above-mentioned hG-CSF DNA fragment with the said vector produces a plasmid pUC19S1SG.

The vector pUC19S1SG was cleaved with Mlu I and BamHI to obtain a DNA fragment (522 bp). The DNA fragment was inserted at the NcoI/BamHI section of the vector pET14b (Novagen, USA) to obtain the vector pT14S1SG. Figure 3 depicts the above procedure for constructing the vector pT14S1SG.

Also, using the plasmid pT14S1SG as a template, through PCR using the SEQ ID Nos. 9 and 10 combining oligonucleotide, the enterotoxin receives a Shine-Dalgarno sequence, and after obtaining the DNA fragment containing the modified hG-CSF gene connected to the enterotoxin signal peptide, an insertion structure is obtained after a restricting it using Xba I and BamHI. On the other hand, a plasmid pT14SS1SG is produced using the cleaved pET14b fragment (Novagen, USA) connected with the above mentioned insertion body. Figure 4 illustrates the above procedure for constructing the vector pT14SS1SG.

Further, treat *E. Coli.* BL21(DE3) bacterial strain with 70 mM calcium chloride; thus, making a confident *E. Coli.*, and also added the plasmid pT14SS1SG suspension into 10 mM of Tris buffer (pH 7.5). Using the resistance and sensitivity to an antibody through a plasmid, he *E. Coli.* transformant HM 10310 was selected through a usual method.

(b) Construction of a hG-CSF expression vector.

Using the published method of site directed mutagenesis, the plasmid pT14SS1SG comprising the serine codon, located on the 1st location of the modified hG-CSF gene, is changed to threonine codon; thus, able to produce an expression vector containing the hG-CSF gene. The plasmid pT14SS1SG is used as a template, and forms a hybrid molecule with a synthetic oligonucleotide

of SEQ ID Nos. 12 and 13 which comprise the modified codon; and beyond this oligonucleotide, it amplifies the gene using four nucleotide triphosphates (ATP, GTP, TTP, CTP) and pfu (Stratagene, USA) which extends the said oligonucleotide in 5' to 3' direction:

	Enterotoxin Signal Peptide				Granulous Colony Inducer					
-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	
Thr	Asn	Ala	Tyr	Ala	Thr	Pro	Leu	Gly	Pro	(SEQ ID No.: 11)
ACA	AAT	GCC	TAC	GCG	ACA	CCC	CTG	GGC	CCT	(SEQ ID No.: 12)
TGT	TTA	CGG	ATG	CGC	TGT	GGG	GAC	CCG	GGA	(SEQ ID No.: 13)

After the amplified gene is recovered, a restriction enzyme Dpn 1 is added to completely remove the plasmid, which was added before the amplification.

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. DNA is recovered from the transformed colony, and by determining the base sequence, the plasmid pT14SSG - where the 1st amino acid serine of hG-CSF was changed to threonine was recovered. Furthermore, using the said plasmid pT14SSG, *E. Coli.* transformant HM 10301 was obtained by transforming the *E. Coli.* BL21 (DE3) bacterial strain using the method outlined in a). By culturing this transformant, the researchers verified the expression of hG-CSF that contained the identical amino acid sequence as that of the wild type.

(c) Construction of a vector containing a gene encoding modified signal peptide.

In order to alter a particular amino acid residue of the enterotoxin signal peptide, an expression vector containing the enterotoxin plasmodium signal peptide was produced using the published site directed mutagenesis method.

First, in order to replace the 4th amino acid of the enterotoxin signal

peptide to threonine, the present inventors used the plasmid pT14SSG and SEQ ID Nos. 15 and 16 oligonucleotide as described in (b) to produce a modified plasmid identical to the one described in (b).

Met Lys Lys **Thr** Ile Ala Phe Leu (SEQ ID NO: 14)
5'-GG-TGT-TTT-ATG-AAA-AAG-**ACA**-ATC-GCA-TTT-CTT-C-3' (SEQ ID NO: 15)
3'-CC-ACA-AAA-TAC-TTT-TTC-**TGT**-TAG-CGT-AAA-GAA-G-5' (SEQ ID NO: 16)

E. Coli. XL-1 blue (Novagen, USA) was transformed by thus obtained plasmid. The base sequence of the DNA recovered from transformed colonies was determined; thus, obtained a plasmid, which contains a gene having threonine in place of the 4th amino acid of the enterotoxin signal peptide. This plasmid was cleaved by XbaI and MluI; thus, obtaining the modified enterotoxin signal peptide insertion body. Furthermore, the plasmid pT14SSG is produced by connecting the above mentioned insertion body with the plasmid vector fragment cleaved by XbaI and MluI.

In order to replace the 22nd amino acid with Gln on the above modified enterotoxin signal peptide, the plasmid pT14SSG-4T produced above and the oligonucleotide of SEQ ID Nos. 18 and 19 was used to produce a modified plasmid through the site directed mutagenesis as mentioned in b).

ASN Ala **Gln** Ala Thr Pro Leu Gly (SEQ ID NO: 17)
5'-CA-AAT-GCC-**CAA**-GCG-ACA-CCC-CTG-GGC-3' (SEQ ID NO: 18)
3'-GT-TTA-CGG-**GTT**-CGC-TGT-GGG-GAC-CCG-5' (SEQ ID NO: 19)

The modified plasmid as obtained above is transformed in *E. Coli.*, then its DNA is recovered, then by determining the base sequence, the 4th amino acid is changed to threonine and the 22nd amino acid is changed to Gln; thus, each modified plasmid is obtained.

In order to alter the Shine-Dalgarno sequence of the modified enterotoxin signal peptide to that of SEQ ID No. 71, the plasmid pT14SSG-4T22Q and the oligonucleotide of SEQ ID No. 20 and 21 produced above are used to produce the modified plasmid through the site directed mutagenesis as mentioned in b). The modified plasmid, as obtained above, is transformed in *E. Coli.* and the DNA is recovered, then obtained the plasmid pT140SSG-4T22Q by determining the base sequence, the Shine Dalgarno sequence of the enterotoxin signal peptide. Figure 5 represents the above procedure for constructing the vector pT140SSG-4T22Q. Using the same method as in a), the *E. Coli.* BL21(DE3) is transformed to obtain the *E. Coli.* transformant HM 10302.

Example 3: Construction of a vector containing a gene encoding modified hG-CSF

To make the hG-CSF gene comprising plasmid, produced from the example 2a), suitable to *E. Coli.* it is changed to a preferred codon of *E. Coli.*, also to prevent the cysteine residue from forming a disulfide bond, the serine residue was altered in the following way.

First, in order to produce the *E. Coli.* preferred codon and hG-CSF gene that include the 17th serine codon, S1 oligomer (SEQ ID No. 22) and AS1 oligomer (SEQ ID No. 23) were synthesized by DNA synthesizer.

Then 0.5 $\mu\ell$ (50 pmole) of each oligomers were taken then reacted under 95℃ for 15 minutes, and then kept it for 3 hours to cool it to 35℃. The mixture was precipitated in ethanol and subjected to gel electrophoresis in a 5% PAGE gel to obtain a cohesive ended double strand oligomer.

On one side, a gene from 4th amino acid to 25th amino acid of G-CSF was deleted by cleaving the plasmid pT14SS1SG with restriction enzymes Apa I and BstX I. This fragment is then ligated with the cohesive ended oligomer to obtain

the plasmid pT14SS1S17SEG. This vector contains a gene encoding hG-CSF having *E. Coli.*-preferred codons at the amino terminus and serine in place of the 1st and 17th amino acids of hG-CSF, respectively. Figure 6 illustrates the above procedure for constructing the plasmid pT140SS1S17SEG.

Using the above plasmid pT14SS1S17SEG *E. Coli.* transformant HM 10311 is produced by transforming the *E. Coli.* BL21(DE3) using the same method as in example 2a). This transformant was deposited with the Korean Culture Center of Microorganisms (KCCM) on March 24, 1999 under the accession number KCCM-10154.

Example 4: Construction of vector containing a gene encoding *E. Coli.* OmpA signal peptide and modified hG-CSF.

A vector containing a gene encoding Tac promoter and OmpA signal peptide (SEQ ID No. 24) as well as a gene encoding modified hG-CSF was prepared as follows:

Met-Lys-Lys-Thr-Ala-Ile-Ala-Ile-Ala-Val-Ala-Leu-Ala-Gly-
Phe-Ala-Thr-Val-Ala-Gln-Ala-Ser-Arg (SEQ ID NO: 24)

--GTT-GCG-CAA-GCT-TCT-CGA-- (SEQ ID NO: 25)

--CAA-CGC-GTT-CGA-AGA-GCT-- (SEQ ID NO: 26)

HindIII restriction site

A vector fragment containing a cohesive end was produced by cleaving the plasmid pFlag.CTS (Eastman, USA), which contains the Tac promoter and OmpA signal peptide using Hind III and EcoR I.

Also, in order to connect the OmpA signal peptide with hG-CSF gene, hG-CSF gene containing plasmid pT-CSF is used as a template. To insert a Hind

III recognition site in the front region of hG-CSF, SEQ ID No. 27 oligomer (designed with serine instead of threonine codon for its 1st amino acid) and SEQ ID No. 28 (designed to insert EcoR I recognition sequence 5'-GAATTC-3' behind the termination codon) are used to obtain a DNA fragment containing hG-CSF encoding arrangement. Then restriction enzymes Hind III and EcoR I cleave so as to produce a hG-CSF DNA fragment containing Hind III and EcoR I ending. Furthermore, the said fragment is connected to the above-mentioned cleaved vector to produce a plasmid pT01SG. Figure 7 illustrates the aforementioned procedure for constructing vector pT01SG.

Using the aforementioned plasmid pT01SG, *E. Coli.* transformant, HM 10409, is produced by transforming *E. Coli.* BL21 (DE3) by using the method as mentioned in the example 2a).

Example 5: Construction of a vector containing a gene encoding OmpA signal peptide and modified hG-CSF.

In order to replace the first codon from serine to threonine in hG-CSF, the plasmid pT01SG and oligonucleotide (SEQ ID Nos. 30 and 31) from the example 4 is used to produce a modified plasmid through the site directed mutagenesis method identical to that of example 2b).

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained plasmid pTOG, which contained a gene having Thr in place of the first amino acid of hG-CSF.

E. Coli. BL21 (DE3) (Stratagene, USA) was transformed with the vector pTOG obtain a transformant designated *E. Coli.* HM 10401.

Example 6: Production of modified hG-CSF

(a) Production of [Ser^1, Ser^{17}] hG-CSF

In order to replace the cysteine codon to serine codon on 17^{th} place of [Ser^1] hG-CSF, the plasmid pTO1SG produced from example 4 and the oligonucleotide of SEQ ID Nos. 32 and 33 was used to produce the modified plasmid through the site directed mutagenesis method identical to that of example 2b).

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined; thus, obtained the plasmid pTO1S17SG, which contained a gene having Ser in place of the 1^{st} and 17^{th} amino acids of hG-CSF.

Further, *E. Coli.* BL21(DE3) (Stratagene, USA) was transformed with the vector pTO1S17SG to obtain a transformant designated *E. Coli.* HM 10410 which was deposited with Korean Culture Center of Microorganisms (KCCM) on March 24, 1999 under accession number KCCM-10151.

(b) Production of [Ser^{17}] hG-CSF

In order to replace the cystein codon with serine codon in the 17^{th} place of pTOG comprising hG-CSF, the plasmid pTOG produced in aforementioned example 5 and the oligonucleotide (SEQ ID Nos. 32 and 33) were used to produce the modified plasmid through the site directed mutagenesis method identical to that in example 2(b).

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined; thus, obtained the plasmid pTO17SG, which contained a gene having Ser in place of the 17^{th} amino acid of hG-CSF.

Further, *E. Coli.* BL21(DE3) (Stratagene, USA) was transformed with the vector pTO17SG to obtain a transformant designated *E. Coli.* HM 10411, which was deposited with the Korean Culture Center of Microorganisms (KCCM) on March 24, 1999 under the accession number KCCM-10152.

(c) Production of [Thr^{17}] hG-CSF

In order to replace the cysteine codon with threonine codon in the 17^{th} place of the pTOG comprising hG-CSF gene, the plasmid pTOG produced in example 5 and the oligonucleotide (SEQ ID Nos. 34 and 35) were used to produce the modified plasmid through the site directed mutagenesis method identical to that of example 2(b).

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. The base sequences of the DNA recovered from transformed colonies was determined; thus, obtained the plasmid pTO17TG which contained a gene having Thr in place of the 17^{th} amino acid of hG-CSF.

E. Coli. BL21(DE3) (Stratagene, USA) was transformed with the vector pTO17TG to obtain a transformed designated *E. Coli.* HM 10413.

(d) Production of [Ala^{17}]hG-CSF

In order to replace the cysteine codon with alanine codon in the 17^{th} place of the pTOG comprising hG-CSF gene, the plasmid pTOG produced in example 5 and the oligonucleotide (SEQ ID Nos. 36 and 37) were used to produce the modified plasmid through the site directed mutagenesis method identical to that of example 2(b).

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. The base sequence of DNA recovered from transformed colonies was determined; thus, obtained the plasmid pTO17AG which contained a gene having

Ala in place of the 17th amino acid of hG-CSF.

E. Coli. BL21 (DE3) (Stratagene, USA) was transformed with vector pTO17AG to obtain a transformant designated *E. Coli.* HM 10414.

(e) Production of [Gly^{17}]hG-CSF

In order to replace the cysteine codon with glycine codon in the 17th place of the pTOG comprising hG-CSF gene, the plasmid pTOG produced in example 5 and the oligonucleotide (SEQ ID Nos. 38 and 39) were used to produce the modified plasmid through the site directed mutagenesis method identical to that of example 2b).

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. The base sequence of DNA recovered from transformed colonies was determined; thus, obtained the plasmid pTO17GG which contained a gene having Gly in place of the 17th amino acid of hG-CSF.

E. Coli. BL21 (DE3) (Stratagene, USA) was transformed with vector pTO17GG to obtain a transformant designated *E. Coli.* HM 10415.

(f) Production of [Asp^{17}]hG-CSF

In order to replace the cysteine codon with Asp codon in the 17th place of the pTOG comprising hG-CSF gene, the plasmid pTOG produced in example 5 and the oligonucleotide (SEQ ID Nos. 40 and 41) were used to produce the modified plasmid through the site directed mutagenesis method identical to that of example 2b).

E. Coli. XL-1 blue (Novagen, USA) was transformed with the modified plasmid. The base sequence of DNA recovered from transformed colonies was determined; thus, obtained the plasmid pTO1APG which contained a gene having Asp in place of the 17th amino acid of hG-CSF.

E. Coli. BL21 (DE3) (Stratagene, USA) was transformed with vector pTO17APG to obtain a transformant designated *E. Coli.* HM-10416.

Example 7: Construction of a vector containing a gene encoding Gene III signal peptide

(a) Construction of a vector containing a gene encoding arabinose promoter and Gene III signal peptide.

A vector containing a gene encoding arabinose promoter and Gene III signal peptide (SEQ ID No. 42) as well as a gene encoding modified hG-CSF was prepared as follows:

Met – Lys – lys – Leu – Leu – Phe – Ala – Ile – Pro – Leu – Val – Val – Pro -
Phe – Tyr – Ser – His - Ser- (SEQ ID No.: 42)

-TAT-AGC-CAT-AGC-ACC-ATG-GAG- (SEQ ID No.: 43)

-ATA-TCG-GTA-TCG-TGG-TAC-CTC- (SEQ ID No.: 44)

Nco I restriction site

After cleaving the arabinose promoter and Gene III signal peptide comprising the plasmid pBAD• gIIIA with the restriction enzyme Nco I, a single stranded DNAs were removed with Klenow DNA polymerase to obtain a blunt-ended double stranded DNA, which was then cleaved with Bgl II to obtain a vector fragment having both blunt end and a cohesive end.

Vector pT-CSF was subjected to PCR using a sense primer (SEQ ID No. 46) having a nucleotide sequence coding for the 2nd to the 9th amino acids of hG-CSF (SEQ ID No. 45) and a complementary antisense primer (SEQ ID No. 47) then it was used to obtain a blunt-ended DNA fragment containing hG-CSF gene

and a BamHI restriction site in the carboxyl terminus. The fragment then was cleaved with BamHI to obtain hG-CSF gene fragment having both a blunt end and a cohesive end.

```
     Pro Leu Gly Pro Ala Ser Ser Leu      (SEQ ID NO 45)
5' -C-CCC-CTG-GGC-CCT-GCC-AGC-TCC-CTG-3'  (SEQ ID NO 46)
3' -G-GGG-GAC-CCG-GGA-CGG-TCG-AGG-GAC-5'  (SEQ ID NO 47)
```

The hG-CSF gene fragment as inserted into the vector obtained above is used to obtain the plasmid pBADG containing the arabinose promoter and Gene III signal peptide. The production process of pBADG and the amino acid sequence (SEQ ID No. 48) of Gene III signal peptide and hG-CSF connection site are illustrated on figure 8.

After transforming *E. Coli.* XL-1 blue with the plasmid obtained above, DNA was recovered from the transformed colony, and the base sequence was determined. Using this pBADG plasmid, the *E. Coli.* transformant HM 10501 was obtained through the method as described in example 2(a). By culturing this transformant, hG-CSF protein expression, that possesses the identical amino acid sequence as its wild type counterpart, was verified.

(b) Production of [Met^2, Val^3] hG-CSF

Plasmid pBAD • gIII A (Invitrogen, USA) was cleaved using restriction enzymes Nco I and Bgl II to produce a vector fragment with cohesive end on both ends.

Met-Lys-Lys-Leu-Leu-Phe-Ala-Ile-Pro-Leu-Val-Val-Pro-
Phe-Tyr-Ser-His-Ser- (SEQ ID NO: 42)
-TAT-AGC-CAT-AGC-ACC-ATG-GAG- (SEQ ID NO: 43)
-ATA-TCG-GTA-TCG-TGG-TAC-CTC- (SEQ ID NO: 44)
NcoI restriction site

On one side, in order to produce hG-CSF gene inserter, pT-CSF plasmid was used as a template. Then, the combined oligonucleotide of SEQ ID Nos 50 and 51 underwent PCR; thus, creating a blunt-ended amino end and a DNA fragment with BamH I recognition site on its carboxyl end. Nco I and BamH I finally recovered this fragment with both of its ends as cohesive ends through restriction.

Thr Met Val Gly Pro Ala Ser Ser Leu (SEQ ID NO: 49)
5'-TAC-GCG-TCC-ATG-GTG-GGC-CCT-GCC-AGC-TCC-CTG-3'(SEQ ID NO: 50)
3'-ATG-CGC-AGG-TAC-CAC-CCG-GGA-CGG-TCG-AGG-GAC-5'(SEQ ID NO: 51)
NcoI restriction site

The hG-CSF gene fragment was inserted into the vector obtained above to obtain vector pBAD2M3VG contained a gene coding *E. Coli.* Gene III signal peptide, and Met and Val in place of the 2nd and 3rd amino acids of hG-CSF (SEQ ID No. 52), respectively. Figure 9 shows the above procedure for constructing the vector pBAD2M3VG.

E. Coli. BL21 (DE3) was transformed with vector pBAD2M3VG to obtain a transformant designated *E. Coli.* HM 10510 according to the method disclosed in Example 2(a), which was deposited with the Korea Culture Center of Microorganisms (KCCM) on March 24, 1999 under accession number of KCCM-10153.

(c) Production of [Ser17] hG-CSF

In order to replace the cysteine codon with a serine codon on the 17th place of hG-CSF gene in the arabinose promoter system, the modified plasmid was produced using the plasmid pBADG as produced in (a) above and the oligonucleotide of SEQ ID Nos. 32 and 33 through the site directed mutagenesis method identical to that in example 2(b).

E. Coli. XL-1 blue was transformed with the modified plasmid. The base sequence of DNA recovered from transformed colonies was determined; thus, obtained the plasmid pBAD17SG which contained a gene having serine in place of the 17th amino acid of hG-CSF.

E. Coli. BL21 (DE3) was transformed with vector pBAD17SG to obtain a transformant designated *E. Coli.* HM 10511 according to the method disclosed in Example 2(a).

(d) Production of [Met2, Val3, Ser17] hG-CSF

From the arabinose promoter system, in order to replace the 2nd and 3rd Lys codons and 17th cysteine codon with methionine, valine, and serine codons respectively, the modified plasmid was produced using pBAD2M3VG plasmid as produced in (b) above and the oligonucleotide of SEQ ID Nos. 32 and 33 through the site directed mutagenesis method as described in example 2(b).

E. Coli. XL-1 blue was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined; thus, obtained the plasmid pBAD2M3V17SG which contained a gene having Met, Val, and Ser in place of the 2nd, 3rd, and 17th amino acids of the hG-CSF, respectively.

E. Coli. BL21 (DE3) was transformed with vector pBAD2M3V17SG to obtain a transformant designated *E. Coli.* HM 10512 according to the method

disclosed in Example 2(a)

.

Example 8: Comparison of hG-CSF Production

The transformants from examples 2 to 7 were each cultured on LB medium, then incubated in the presence of an expression inducer (IPTG) for 3 hours or cultured in absence of IPTG for more than 15 hours. Each of the cultures was centrifuged, then the clumps were treated according to the Osmotic Pressure Shock Method (Nossal, G.N., *J. Biol. Chem.*, 241, 3055 (1966)). Specifically, they were centrifuged at 6000 rpm for 20 minutes to precipitate bacterial cells, and the precipitate was suspended in a 1/10 volume of isotonic solution (20% sucrose, 10mM Tris-Cl buffer solution containing 1mM EDTA, pH 7.0). The suspension was allowed to stand for 30 minutes, then centrifuged again to collect the bacterial cells. Next, the collected cells were resuspended in distilled water at 4℃; thus, extracted protein existing inside the cells' periplasm and obtained a supernatant as a periplasmic solution. The hG-CSF level in the periplasmic supernatant was assayed in accordance with the Enzyme-Linked Immuno-Sorbent Assay (ELISA) method using an antibody against hG-CSF (Aland, USA), which was calculated as the amount of hG-CSF produced per 1ℓ of culture. The results are shown in Table 1.

Table 1

Comparison of hG-CSF and modified hG-CSF productions

Transformant	Example	Expression Vector	CSF Amino Acid Residue	hG-CSF Level in Periplasm (mg/ℓ)
HM 10301	2(b)	pT14SSG		65
HM 10302	2(c)	pT140SSG-4T22Q		277
HM 10310	2(a)	pT14SS1SG	Ser^{1}	92
HM 10311	3	PT14SS1S17SEG	Ser^{1}, Ser^{17}	1512
HM 10401	5	PTOG		85
HM 10409	4	pTO1SG	Ser^{1}	105
HM 10410	6(a)	pTO1S17SG	Ser^{1}, Ser^{17}	1477
HM 10411	6(b)	pTO17SG	Ser^{17}	1550
HM 10413	6(c)	pTO17TG	Thr^{17}	1373
HM 10414	6(d)	pTO17AG	Ala^{17}	1486
HM 10415	6(e)	pTO17GG	Gly^{17}	1480
HM 10416	6(f)	pTO17APG	Asp^{17}	67
HM 10501	7(a)	pBADG		54
HM 10510	7(b)	pBAD2M3VG	Met^{2}, Val^{3}	69
HM 10511	7(c)	pBAD17SG	Ser^{17}	937
HM 10512	7(d)	pBAD2M3V17SG	Met^{2}, Val^{3}, Ser^{17}	983

Example 9: Post-treatment and Purification

Transformant *E. Coli.* HM 10411 was cultured, then the cultured solution was centrifuged at 6000 rpm for 20 minutes to harvest cells. The periplasm fraction was obtained using the Osmotic Pressure Shocking Method.

These fractions were each purified using a column. Ion exchange resin, absorption, and gel filtration column or antibody column are appropriate vehicle for this. The periplasmic solution was adjusted to pH 5.0 to 5.5, then absorbed on a CM-Separose (Pharmacia Inc., Sweden) column pre-equilibrated to pH 5.3, then the column was washed with 25 mM NaCl. hG-CSF was eluted by sequentially adding to the column buffer solutions containing 50 mM, 100 mM, and 200 mM NaCl, and fractions containing hG-CSF were collected and combined. Then, the combined fractions were subjected to Phenyl Separose (Pharmacia Inc., Sweden) column chromatography to obtain [Ser^{17}]hG-CSF having a purity of 99%. Also, the above procedure was repeated using each of the transformants *E. Coli.* HM 10311, HM 10409, HM 10410, HM 10413, HM 10414, HM 10415, HM 10510, and HM 10512, respectively. The concentration and purity of the purified hG-CSF were analyzed using the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) or HPLC, then subjected to ELISA method, as described in example 8, to determine the exact hG-CSF concentration in the periplasmic solution. Further, as the result of analyzing 32 amino end sequences, the sequences were verified to be normal sequences that lacks methionine. Also, the 1^{st}, 2^{nd}, 3^{rd}, or 17^{th} amino acids were found to have been replaced at an exact location on the amino end of hG-CSF. The sequences were SEQ ID Nos. 56, 58, 60, 62, 64, 66, 68, and 70, respectively.

Example 10: A Comparison of the Protein level produced from the recombinant strain

The expression and the production level of hG-CSF from the recombinant strain was verified using SDS-PAGE and Western blotting.

First, the periplasm fraction of E. coli transformant obtained in example 9 and the purified [Ser^{17}] hG-CSF were analyzed using Met-hG-CSF

(Kirin Amgen) as the control group through SDS-PAGE. Figure 10a represents the SDS-PAGE result, wherein lane 1, Met-G-CSF control; lane 2, the periplasm fraction of the transformant *E. Coli.* HM 10411, and lane 3, the purified [Ser^{17}] hG-CSF. As shown in result, it was verified that [Ser^{17}] hG-CSF havs the same molecular weight as the wild-type hG-CSF, and that the periplasmic solution of the transformant *E. coli* HM 10411 contains a high level of [Ser^{17}] hG-CSF.

A nitrocellulose filter (Bio-Rad Lab., USA) was wetted with a buffer solution for blotting (170 mM glycine, 25 mM Tris • HCl (pH8), 20% methanol) and the proteins separated on the gel were Western blotted onto a nitrocellulose filter (Bio-Rad Lab., USA) for 3 hours. The filter was kept in 1% Casein for 1 hour and then washed three times wit hPBS containing 0.05% Tween 20. The filter was put in a goat anti-G-CSF antibody (R&D System, AB-214-NA, USA) solution diluted with PBS and reacted at room temperature for 2 hours. After the reaction, the filter was washed 3 times with a PBST solution to remove un-reacted antibody. Horseradish peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad Lab., USA) diluted with PBS was added thereto and reacted at the room temperature for 2 hours. The filter was washed with PBST, and a peroxidase substance kit (Bio-Rad Lab., USA) solution was added thereto to develop a color reaction. The results from the above Western blotting are shown in figure 10b, wherein lane 1 represents a positive control, Met-G-CSF, and lane 2 represents the purified [Ser^{17}] hG-CSF.

As can be seen from figure 10b, the molecular weight of [Ser^{17}] hG-CSF equals that of the wild type hG-CSF.

Example 11: Cellular activity of hG-CSF and the modified hG-CSF

The cell line HL-60 (ATCC CCL-240 derived from the bone marrow of a promyelocytic leukemia patient / a 36 year-old Caucasian woman) was cultured

in RPMI 1640 media containing 10% fetal bovine serum and adjusted to 2.2 X 10^5 cells/mℓ, followed by adding thereto DMSO (dimethylsulfoxide, culture grade/SIGMA) to a concentration of 1.25% (v/v). 90 μℓ of the resulting solution was added to a 96 well plate (Corning/low evaporation 96 well plate) in an amount of 2 X 10^4 cells/well and incubated at 37℃ under 5% CO_2 for 48 hours.

Each of the modified hG-CSF was diluted in RPMI 1640 media to a concentration of 500 ng/mℓ and then serially diluted 10 times by 2-fold with RPMI 1640 media. As a positive control, a commercially available hG-CSF(Jeil Pharmaceutical.).

The resulting solution was added to wells of HL-60 cell line at 10 μℓ per well to consequently reduce from a concentration of 50 ng/mℓ and incubated at 37 ℃ for 48 hours.

The level of cell line increased was determined using a commercially available CellTiter96TM (Cat# G4100, Promega) based on the measured optical density at 670nm.

As can be seen from figure 11, the cellular activities of the modified hG-CSFs are the same as, or higher than that of the positive control, wild type hG-CSF. Specifically, the activity of the wild type and that of [Thr^{17}] hG-CSF are approximately the same, but the activity of [Ser^{17}] hG-CSF and [Gly^{17}] hG-CSF are higher than that of the wild type. Accordingly, the biological activities of hG-CSFs of the present example are equal if not higher than that of the wild type.

Effect of Invention

The modified hG-CSFs of the present invention are superior in its expression level, also when expressed in combination with a signal peptide they show a superior secretion level. Also, the hG-CSF or modified hG-CSF having

no added methionine residue at its N-terminus can be prepared by expressing the hG-CSF or the modified hG-CSF connected to a signal peptide of the present invention hG-CSF to periplasm.

What is claimed is:

1. A modified human granulocyte-colony stimulating factor (hG-CSF) which is characterized in that at least one of the 1st, 2nd, 3rd and 17th amino acids of the wild type hG-CSF (SEQ ID No. 2) is replaced by other amino acid(s).

2. The modified hG-CSF of claim 1 whose amino acid sequence is the same as that of the wild type hG-CSF, except that

the 1st and 17th amino acids are replaced by other amino acids, respectively;

the 2nd and the 3rd amino acids are replaced by other amino acids, respectively;

the 2nd, 3rd and 17th amino acid are replaced by other amino acids, respectively; or

the 17th amino acid is replaced by other amino acid.

3. The modified hG-CSF of claim 1 whose amino acid sequence is the same as that of the wild type hG-CSF, except that

the 1st amino acid and 17th amino acid are replaced by Cys and Ser, respectively;

the 2nd amino acid to Met and 3rd amino acid to Val;

the 2nd amino acid to Met, 3rd amino acid to Val, and 17th amino acid to Ser; or

the 17th amino acid to Ser, Thr, Ala, or Gly.

4. DNA encoding the modified hG-CSF of the claim 1.

5. The DNA of claim 4, wherein the 1st to the 96th base sequences correspond to one selected from the group consisting of SEQ ID Nos. 55, 57, 59, 61, 63, 65, 67, and 69 and the 97th to the remaining base sequence are the same as that of the wild type hG-CSF.

6. A modified hG-CSF expression vector comprising the DNA in claim 4.

7. The modified hG-CSF expression vector of claim 6, which further comprises a polynucleotide encoding a signal peptide.

8. The modified hG-CSF expression vector of claim 6, which is pT14SS1SG, pT14SS1S17SEG, pT01SG, pT01S17SG, pT017SG, pT017TG, pT017AG, pT017GG, pBAD2M3VG, pBAD17SG, or pBAD2M3V17SG.

9. A microorganism transformed with the modified hG-CSF expression vector according to claim 6.

10. The microorganism of the claim 9, which is a transformed *E. Coli*.

11. The microorganism of the claim 10, wherein the transformed *E. Coli* is *E. Coli* BL21(DE3)/pT14SS1SG (HM 10310); *E. Coli* BL21(DE3)/pT14SS1S17SEG (HM 10311; KCCM-10154); *E. Coli* BL21(DE3)/pT01SG (HM 10409); *E. Coli* BL21(DE3)/pT01S17SG (HM 10410; KCCM-10151); *E. Coli* BL21(DE3)/pT017SG (HM 10411; KCCM-10152); *E. Coli* BL21(DE3)/pT017TG (HM 10413); *E. Coli* BL21(DE3)/pT017AG (HM 10414); *E. Coli* BL21(DE3)/pT017GG (HM 10415); *E. Coli* BL21(DE3)/pBAD2M3VG (HM 10510; KCCM-10153); *E. Coli*

BL21(DE3)/pBAD17SG (HM 10511); or *E. Coli* BL21(DE3)/pBAD2M3V17SG (HM 10512).

12. A process for producing a modified hG-CSF having no added methionine residue at its N-terminus which comprises the steps of transforming *E. Coli* with the vector comprising the gene encoding the modified hG-CSF and a gene encoding signal peptide attached at 5'-end thereof; and culturing the transformed *E. Coli* under an appropriate condition to *E. coli* periplasm .

13. The process of claim 12, wherein the modified hG-CSF is characterized in that at least one of the 1^{st}, 2^{nd}, 3^{rd}, and 17^{th} amino acids of the wild type hG-CSF (SEQ ID No. 2) is replaced by other amino acid(s).

14. The process of claim 12, wherein the modified hG-CSF whose amino acid sequence is the same as that of the wild type hG-CSF, except that

the 1st amino acid Thr and 17th amino acid Cys are replaced by other amino acids, respectively;

the 2nd amino acid Pro and 3rd amino acid Lys are replace by other amino acids, respectively;

the 2nd amino acid Pro, 3rd amino acid Lys, and 17th amino acid Cys are replaced by other amino acids, respectively; and

the 17th amino acid Cys is replaced by other amino acid.

15. The process of claim 12, wherein the modified hG-CSF whose amino acid sequence is the same as that of the wild type hG-CSF, except that

the 1^{st} amino acid and 17^{th} amino acid are replaced by Cys and Ser, respectively;

the 2nd amino acid to Met and 3rd amino acid to Val;

the 2nd amino acid to Met, 3rd amino acid to Val, and 17th amino acid to Ser; or

the 17th amino acid to Ser, Thr, Ala, or Gly.

16. The process of claim 12, wherein the hG-CSF encoding gene whose the 1st to the 96th base sequences correspond to one selected from the group consisting of SEQ ID Nos. 55, 57, 59, 61, 63, 65, 67, and 69 and the 97th to the remaining base sequence are the same as that of the wild type hG-CSF.

17. The process of claim 12, wherein the signal peptide is *E. Coli* thermoresistant enterotoxin signal peptide or modified *E. Coli* thermoresistant enterotoxin signal peptide.

18. The process of claim 17, wherein the *E. Coli* thermoresistant enterotoxin signal peptide has the amino acid sequence of SEQ ID No: 53.

19. The process of claim 17, wherein t he *E. Coli* thermoresistant enterotoxin signal peptide has the amino acid sequence of SEQ ID No: 54.

20. The process of claim 17, which further comprises a modified *E. Coli* thermoresistant enterotoxin Shine-Dalgarno sequence having the nucleotide sequence of SEQ ID No: 71.

21. The process of the claim 12, wherein the signal peptide is *E. Coli* beta lactamase signal peptide or modified *E. Coli* beta lactamase signal peptide.

22. The process of the claim 21, wherein the *E. Coli* beta lactamase signal peptide has the amino acid sequence of SEQ ID No: 24.

23. The process of the claim 12, wherein the signal peptide is *E. Coli* Gene III signal peptide or modified *E. Coli* Gene III signal peptide.

24. The process of the claim 23, wherein the *E. Coli* Gene III signal peptide has the amino acid sequence of SEQ ID No: 42.

25. The process of the claim 12, wherein the transformed *E. Coli* is *E. Coli* BL21(DE3)/pT14SS1SG (HM 10310); *E. Coli* BL21(DE3)/pT14SS1S17SEG (HM 10311; KCCM-10154); *E. Coli* BL21(DE3)/pT01SG (HM 10409); *E. Coli* BL21(DE3)/pT01S17SG (HM 10410; KCCM-10151); *E. Coli* BL21(DE3)/pT017SG (HM 10411; KCCM-10152); *E. Coli* BL21(DE3)/pT017TG (HM 10413); *E. Coli* BL21(DE3)/pT017AG (HM 10414); *E. Coli* BL21(DE3)/pT017GG (HM 10415); *E. Coli* BL21(DE3)/pBAD2M3VG (HM 10510; KCCM-10153); *E. Coli* BL21(DE3)/pBAD17SG (HM 10511); or *E. Coli* BL21(DE3)/pBAD2M3V17SG (HM 10512).